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Myriocin prevents fumonisin B_1-induced sphingoid base accumulation in mice liver without ameliorating hepatotoxicity

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Abstract

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides* present on corn and corn-based products, causes species- and organ-specific diseases. The hepatotoxic effects of FB_1 in mice have been closely correlated with the accumulation of free sphinganine, a marker for ceramide synthase inhibition, and reduced biosynthesis of more complex sphingolipids. It has been shown that FB_1 modulates expression of many cell signaling factors. In the current study we used myriocin, a specific inhibitor of serine palmitoyltransferase, to investigate the role of free sphinganine accumulation in FB_1-induced hepatotoxicity and increased expression of selected signaling genes in BALB/c mice. The mice were pretreated daily with intraperitoneal injection of 1.0 mg/kg myriocin 30 min before subcutaneous injections of 2.25 mg/kg of FB_1 for 3 days. Results showed that myriocin alone was not hepatotoxic and the combination of myriocin plus FB_1 completely prevented the FB_1-induced elevation of hepatic free sphinganine and prevented the FB_1-induced induction of selected cell signaling genes, suggesting that accumulation of free sphinganine and/or its metabolites contribute to the FB_1-modulation of the cell signaling factors. However, the combination of myriocin and FB_1 did not prevent FB_1-increased concentration of plasma alanine aminotransferase and only slightly attenuated aspartate aminotransferase; it did not affect the FB_1-induced hepatocyte apoptosis or increased cell proliferation. A longer combined treatment of myriocin and FB_1 was highly toxic. The hepatotoxic effects in mice seen in this study are most likely due to a combination of factors including accumulation of free sphinganine, depletion of more complex sphingolipids and sphingomyelin, or other unknown mechanisms.
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Keywords: Fumonisin; Myriocin; Cytokine expression; Hepatotoxicity; Sphinganine; Sphingolipids

1. Introduction

Fumonisins were first isolated in 1988 from the fungus *Fusarium verticillioides*, a common endophytic fungus in corn (Gelderblom et al., 1988). Several types of fumonisins have been identified so far as products of *F. verticillioides* in naturally contaminated corn and corn-based products. Fumonisin B_1 (FB_1) is the most abundant and most toxic of all fumonisin isomers investigated so far (WHO, 2000). Fumonisin B_1 induces species-specific toxicity. In horses this toxin is known to cause leukoencephalomalacia (Marasas, 2001); in pigs pulmonary edema and cardiovascular damage (Haschek et al., 2001; Smith et al., 1999). The high incidence of esophageal cancer in people in some areas of South Africa and China was correlated with *F. verticillioides* infection and fumonisin levels in corn (Marasas, 2001; Yoshizawa et al., 1994). It has been demonstrated that FB_1 is hepato- and nephro-carcinogenic in male rats (Gelderblom et al., 1991; Howard et al., 2001), and hepatocarcinogenic in female mice (Howard et al., 2001). Fumonisins are hepatotoxic and nephrotoxic in

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rodents (Sharma et al., 1997; Voss et al., 1998, 2001). The cellular effects of fumonisins consist of a mixture of apoptosis and necrosis and regenerative proliferation (Lemmer et al., 1999; Howard et al., 2001; Sharma et al., 1997).

Fumonisins are structurally similar to free sphingoid bases (sphinganine and sphingosine), and inhibit ceramide synthase (sphingosine *N*-acyltransferase), a critical enzyme in the pathway of de novo sphingolipid synthesis (Merrill et al., 2001; Wang et al., 1991). By inhibiting ceramide synthase, FB_1 increases the level of free sphinganine in tissues, serum, and urine (Riley et al., 1993, 1996, 1997; Wang et al., 1992, 1999), decreases complex sphingolipids (Wang et al., 1992; Yoo et al., 1996), and increases the formation of other lipid metabolites such as sphingoid base-1-phosphates and their downstream metabolites (Merrill et al., 2001; Smith and Merrill, 1995). The hepatotoxicity of FB_1 is closely correlated with the accumulation of free sphinganine in male BALB/c (Tsunoda et al., 1998) and other mouse strains (Riley et al., 2001).

Serine palmitoyltransferase (SPT), the first enzyme in the pathway for de novo biosynthesis of sphingolipids, catalytically incorporates L-serine into palmitoyl-coenzyme A to produce 3-keto-sphinganine, the immediate precursor of sphinganine (Hannun et al., 2001, Fig. 1). Inhibition of SPT to reduce free sphinganine accumulation reversed FB_1 toxicity in mammalian cell cultures (He et al., 2002a; Riley et al., 1999a,b; Schmelz et al., 1998; Tolleson et al., 1999; Yoo et al., 1996). Myriocin, a selective inhibitor of SPT (Miyake et al., 1995), prevented accumulation of free sphinganine in kidney of mice exposed to FB_1, and therefore it was proposed that myriocin might be useful in protecting against FB_1 toxicity in vivo (He et al., 2002a; Riley et al., 1999a).

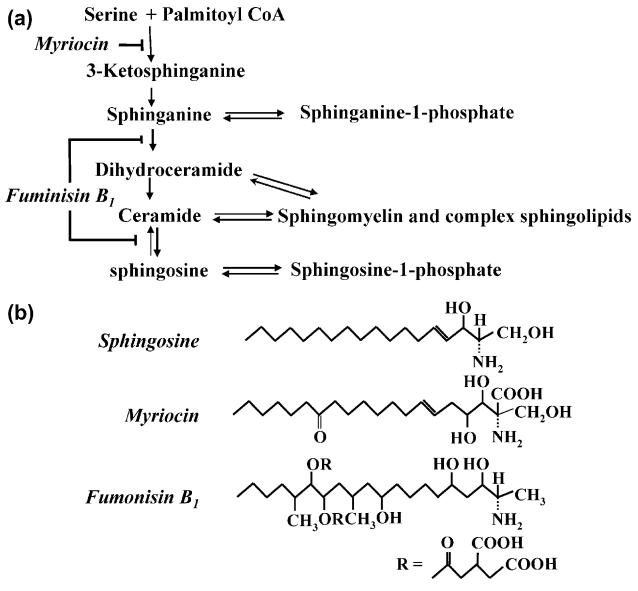

Fig. 1. (a) Sphingolipid pathways indicating inhibition of serine palmitoyltransferase by myriocin and ceramide synthase by fumonisin B_1. Treatment of animals with myriocin decreases synthesis of all sphingolipids, whereas treatment with FB_1 results in the increase of sphinganine, sphingosine and their respective phosphates along with decrease in ceramide and complex sphingolipids. (b) Structural similarity of myriocin and fumonisin B_1 with free sphingoid base sphingosine.

Numerous studies have shown that FB_1 modulates the expression of inflammatory cytokines and other cell signaling factors. For example, FB_1 treatment induced the expression of tumor necrosis factor (TNF) and pro-apoptotic signaling genes in liver and kidney of mice (Bhandari and Sharma, 2002; Bhandari et al., 2002). Peritoneal macrophages from FB_1-treated mice produced higher amount of TNFα than those from saline controls in response to lipopolysaccharide ex vivo (Dugyala et al., 1998). Treatment of LLC-PK_1 cells, a pig renal epithelial cell line, with FB_1 transiently increased the expression of TNFα but this induction of TNFα by FB_1 was unaltered when free sphinganine accumulation was prevented in the cultures by myriocin (He et al., 2001). Expression of TNFα receptor-associated protein (TRAP) 2 was induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS cells (Zhang et al., 2001). It remains uncertain whether FB_1-induced alterations of gene expression in tissues are due solely to the disruption of sphingolipid metabolism.

Myriocin is able to inhibit the activity of hepatic SPT in mice (He et al., 2004a), and subsequently prevent the accumulation of free sphinganine in response to FB_1 both in vivo and in vitro (Enongene et al., 2002; Riley et al., 1999a; Schmelz et al., 1998). It has been widely used to study the role of sphinganine and de novo generated ceramide in regulation of cell functions under various conditions (He et al., 2001, 2002a,b; Le Stunff et al., 2002; Riley et al., 1999a; Schmelz et al., 1998). In the present study, we investigated the effect of myriocin on FB_1 hepatotoxicity and gene expression of selected cytokines in mice. Myriocin effectively blocked the activity of SPT and prevented the FB_1-accumulation of free sphinganine in both liver and kidney; however, it did not reduce FB_1-induced hepatocyte apoptosis or increased proliferating cell nuclear antigen (PCNA) containing cells. The FB_1-induced plasma alanine aminotransferase (ALT) was unaltered by myriocin pretreatment; the elevation of plasma aspartate aminotransferase (AST) was significantly reduced. In spite of minimal protection against FB_1-induced hepatotoxicity, myriocin reversed the FB_1-induced increases in the expression of *TNFα*; *TNF related apoptosis-inducing ligand* (*TRAIL*), *TNFα receptor* (*TNFR*)1, *lymphotoxin* (*LT*)β, *Interferon* (*IFN*)γ, and *transforming growth factor* (*TGF*)β1. Results suggested that elevation in free sphinganine and/or its metabolites are involved in FB_1-induced alterations in expression of various cell signaling factors. However, with the dosing regime used in

this study, myriocin did not provide protection from FB_1-induced hepatotoxicity and prolonged exposure to the combination of the two inhibitors of de novo sphingolipid biosynthesis (FB_1 and myriocin) could significantly potentiate the toxicity of either compound.

2. Materials and methods

2.1. Chemicals

Fumonisin B_1 (>98% purity) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (Tygerberg, South Africa). Myriocin, (2S,3R,4R, 6E-2-amino-3,4-dihydroxy-2-hydroxymethyl-14-oxo-6-eicosenoic acid) was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). C_{20}-sphinganine standard (D-*erythro*-C_{20}-dihydrosphingosine, 98% purity) was obtained from Matreya Inc. (Pleasant Gap, PA, USA). All other reagents were purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA), unless otherwise stated.

2.2. Animal treatments and sampling

Six-week-old female Balb/c mice weighing about 22 g were procured from Harlan Laboratories (Indianapolis, IN). Females were employed as they are more sensitive to FB_1-induced hepatotoxicity than males (Bhandari et al., 2001). They were acclimatized for one week before dosing under targeted environmental conditions of 23 °C and 65% relative humidity, with a 12 h light/dark cycle. Feed and water were provided ad libitum. Animals were treated with humane care following the Public Health Service Policy on Humane Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee.

Mice were divided randomly into four groups with five animals each. Treatment with myriocin was via intraperitoneal route (1.0 mg/kg body weight/day), whereas FB_1 (2.25 mg/kg body weight/day) was injected subcutaneously. Treatment groups received sterilized phosphate buffered saline (PBS) only, myriocin in PBS, FB_1 in PBS, or myriocin and then 30 min later FB_1. In a preliminary experiment mice were treated for 5 days; myriocin alone (1 mg/kg body weight/day) caused significant elevation of serum ALT and AST and the combination of myriocin and FB_1 was lethal; no overt visual signs of toxicity were observed in three days treatment. Therefore, in the subsequent experiment, the mice were treated with only three daily injections. The FB_1 treatment protocol has been proven to produce consistent liver damage in female mice exposed to FB_1 in our laboratory (He et al., 2004b).

One day following the final FB_1 treatment, mice were sacrificed by decapitation. Blood was collected in heparinized tubes and plasma was subsequently isolated for ALT and AST analyses. Livers and kidneys were collected from each animal and weighed; aliquots were fixed immediately in 10% neutral formaldehyde, or frozen in liquid nitrogen and stored at −85 °C until analysis. Body weights were recorded during the treatment period.

2.3. Evaluation of hepatotoxicity

Activities of circulating ALT and AST were determined by using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN). The methods used for determination of the activities of these enzymes are based on a kinetic reduction of β-nicotinamide adenine dinucleotide (reduced disodium salt hydrate) in the reactions detected photometrically. The assays conform to the standard procedures recommended by the International Federation of Clinical Chemistry (Bergmeyer et al., 1986a,b).

Hepatocyte apoptosis was analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay as described previously (Sharma et al., 2003). Liver sections (5 μm) were subjected to dUTP nick-end labeling by TdT with a peroxidase-based in situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN). The TUNEL-positive cells were counted and normalized to the unit area (Sharma et al., 1997).

2.4. Immunohistochemistry for proliferating cellular nuclear antigen (PCNA) assay

Hepatocyte proliferation was estimated by PCNA staining in liver tissue sections as described previously (Sharma et al., 2003). Liver sections (5 μm) were hydrated followed by antigen retrieval in boiling citric acid buffer for 25 min. The primary anti-PCNA antibodies were incubated overnight with the sections at 4 °C in a humidified chamber. The secondary antibodies were applied and the sections were stained with Vectorstain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). The number of PCNA-positive cells were counted and normalized to the unit area.

2.5. Activity of serine palmitoyltransferase (SPT)

The activity of SPT in liver and kidney was analyzed using the method described previously (Williams et al., 1984) with minor modifications. Briefly, the frozen tissues were homogenized in homogenization buffer (50 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], 5 mM DL-dithiothreitol, 10 mM ethylenediaminetetraacetic acid, 0.25 M sucrose, pH 7.4), and the homogenate centrifuged at 30,000*g* for 30 min. Aliquots of 100 μg protein in the supernatant were used for

analysis of SPT activity as described previously (He et al., 2004a; Williams et al., 1984). Bradford (Bio-Rad Laboratories, Hercules, CA, USA) reagent was used to determine the protein content.

2.6. Sphingolipid analysis by high performance liquid chromatography (HPLC)

Free sphingoid bases or complex sphingolipids were determined in base-treated or acid-treated lipid extracts by HPLC utilizing the extraction methods described previously (Riley et al., 1999b). Sphingoid bases were quantified based on the recovery of a C_{20}-sphinganine standard. The HPLC apparatus and derivation procedure were similar to those indicated earlier (He et al., 2001, 2004b).

2.7. Sphingomyelin assay in the liver by fluorescence-based enzyme-catalyzed method

Sphingomyelin was analyzed by a fluorescence-based method following enzyme catalysis (He et al., 2002b). Briefly, the livers were homogenized in 0.25% Triton X-100 in PBS (at a ratio of 1:20 g/ml) and centrifuged (10,000*g* for 5 min). Aliquots of 20 μl supernatant were mixed with equal volumes of homogenizing buffer followed by heating at 70 °C for 5 min; the heated samples were cooled to room temperature and briefly centrifuged. Ten microliters of the supernatant was added to an enzyme cocktail consisting of 12.5 mU of *Bacillus cereus* sphingomyelinase, 400 mU of alkaline phosphatase, 120 mU choline oxidase, 200 mU of horseradish peroxidase, and 20 nmol of 10-acetyl-3-dihydrophenoxazine, a sensitive fluorogenic probe for hydrogen peroxide (Amplex® red reagent, Molecular Probes, Inc., Eugene, OR, USA) in reaction buffer. For each sample, a negative control containing 10 μl of the sample and the same reaction mixture without sphingomyelinase was employed. After 20 min incubation at 37 °C, the microtiter plate was read using a fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The excitation and emission wavelengths were set to 560 and 590 nm, respectively. Sphingomyelin contents were calculated from the difference in fluorescence between the test and the negative control samples by comparing with a sphingomyelin standard curve.

2.8. Ribonuclease protection assay (RPA) for selected gene expression

Total RNA from liver tissue was extracted with TRI® reagents (Molecular Research Center, Cincinnati, OH). An aliquot part of 50 μg RNA was used for RPA using RiboQuant™ RPA starter kit (BD Biosciences, San Diego, CA, USA) as recently described (Sharma et al., 2003). Briefly, high specific-activity α-^{32} P-UTP-labeled anti-sense RNA probes were synthesized using T7 RNA polymerase in vitro transcription kit according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). The synthesized probes were hybridized overnight with RNA at 65 °C followed by treatment with RNase A and T1, and then protease K. The RNase-protected products were extracted and resolved on gels containing 5% polyacrylamide/7 M urea. The α-^{32}P-labeled bands were exposed to an FX Imaging Screen K-HD® (Bio-Rad Laboratories, Hercules, CA, USA) for 6–24 h and scanned by Bio-Rad Molecular Imager® FX. The relative gene expression was digitized using the Quantity one® software (Bio-Rad Laboratories) and normalized against ribosomal protein L32, the housekeeping gene.

2.9. Statistical analysis

Results are presented as mean ± standard error (SE). Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range tests unless otherwise stated in the text. All statistical analyses were done using the SAS program (SAS, Cary, NC, USA). The level of $p < 0.05$ was considered significant.

3. Results

3.1. Toxicity of FB_1 after pretreatment of mice with myriocin

In an early experiment when the treatment was followed for five days the mice treated with either FB_1 or myriocin did not exhibit any behavioral abnormality; however, all of the animals treated with FB_1 + myriocin died within 12 h of the final injection (Fig. 2). The animals did not show any gross abnormality until the

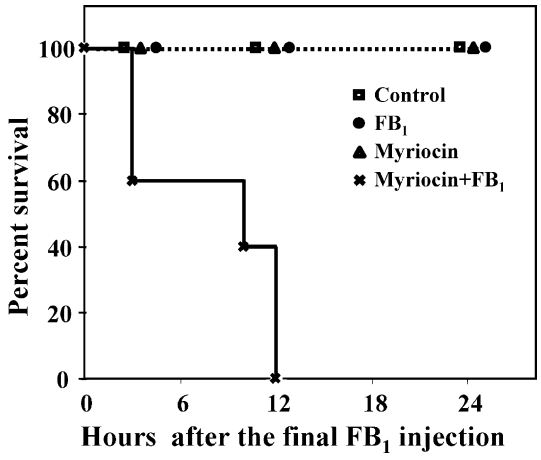

Fig. 2. Survival of mice after five-day treatments with FB_1 or/and myriocin.

fourth day of the treatment but were severely depressed after the injections on this day. These animals had reduced locomotor activity and did not consume any food or water. The treatment with FB_1 or myriocin resulted in increased circulating ALT and AST after five days (data not presented).

During three daily treatments, no obvious behavioral changes were observed in any of the treatment groups. Myriocin treatment alone did not change body weights compared to saline control. Treatment of mice with FB_1 or myriocin + FB_1 significantly reduced their body weights to a similar degree (data not shown). There were no significant differences in absolute or relative livers weights (compared to body weights) among treatments (data not shown). Treatment of mice with myriocin alone for three days did not show alteration in the activities of circulating ALT and AST (Fig. 3); treatment with FB_1 alone or FB_1 + myriocin caused a significant increase in both plasma ALT and AST. Plasma ALT was not reduced in the FB_1 + myriocin group compared to FB_1 alone, whereas the increase in plasma AST was slightly but significantly decreased in the FB_1 + myriocin group compared to FB_1 alone (Fig. 3).

As expected, the FB_1 treatment caused a significant increase in apoptotic cells in the liver (Table 1). There was no evidence of ongoing apoptosis in the liver of either the control or myriocin-treated mice; however, the number of apoptotic cells in the liver of mice treated with myriocin + FB_1 was similar to FB_1 alone (Table 1). The number of PCNA-positive cells in livers of mice treated with FB_1 alone or myriocin alone was similar and although markedly greater than in the controls, the increase was not statistically significant due to a large variability. The combination of FB_1 + myriocin appeared to have an additive effect on the number of PCNA-positive cells compared to FB_1 or myriocin; however, the differences were not statistically significant. No attempt was made to determine if the increased PCNA staining was due to cells being arrested in specific stage of the cell cycle.

3.2. Myriocin blocked activity of serine palmitoyltransferase (SPT)

The activity of SPT in the liver, but not in kidneys was significantly increased following FB_1 treatment compared to that of saline-treated controls (Fig. 4). Consistent with our recently published work (He et al., 2004a), myriocin reduced the SPT activity to 30% of control in the liver, and partially blocked FB_1-induced activation of SPT.

3.3. Myriocin prevented FB_1-induced accumulation of free sphinganine and inhibited the biosynthesis of complex sphingolipids

Treatment with FB_1 significantly increased the concentrations of free sphinganine in both liver and kidney (Fig. 5). The concentration of free sphingosine in both liver and kidney was significantly reduced by myriocin. The FB_1-induced accumulation of free sphinganine in the liver was effectively blocked by co-treatment with myriocin but that of sphingosine was only slightly reduced compared to FB_1 alone.

The concentrations of complex sphingolipids containing sphingosine in the liver were significantly reduced by FB_1 or myriocin; these were further decreased by the combination of myriocin plus FB_1 (Table 2). The level of complex sphingolipids containing sphinganine was decreased by myriocin or myriocin + FB_1; the overall concentrations of complex sphingolipids containing sphingosine or sphinganine were lower in the liver of animals given FB_1 and myriocin together. The hepatic sphingomyelin was significantly

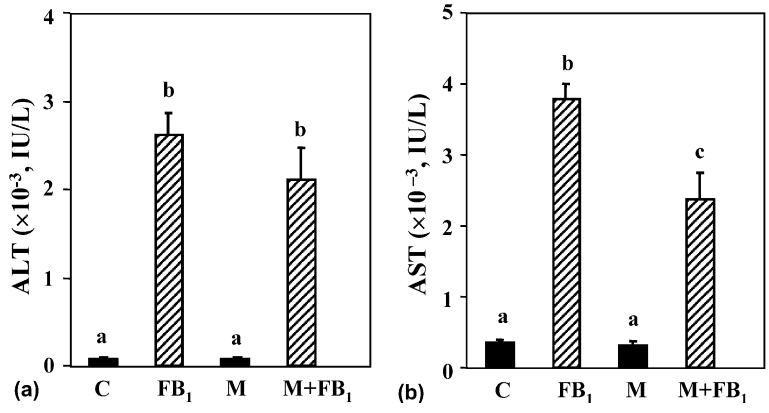

Fig. 3. Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced increase of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Female BALB/c mice were daily treated with 1.0 mg/kg myriocin intraperitoneally, and/or 2.25 mg/kg FB_1 subcutaneously for 3 days. One day after the last FB_1 treatment, the animals were sacrificed and plasma was used for analysis of ALT and AST. Mean ± SE ($n = 5$). Different letters indicate statistical difference at $p < 0.05$.

Table 1
Number of apoptotic and proliferating hepatocytes after myriocin and FB_1 treatment[a]

Treatment	Animal number	Apoptosis incidence	Apoptotic cells/cm^2	Proliferation incidence	Proliferating cells/cm^2
Control	5	0/5	0	3/5	3.8 ± 2.6
FB_1	5	5/5	49.1 ± 20.0*	2/5	69.0 ± 51.4
Myriocin	5	0/5	0	5/5	68.1 ± 29.7
Myriocin + FB_1	5	5/5	41.4 ± 14.3*	5/5	189.6 ± 94.4

*$p < 0.05$ vs. control.
[a] Cell apoptosis and proliferation were analyzed in liver sections by TUNEL assay and PCNA immunohistochemistry, respectively, and expressed per unit area. The positively stained cells were counted under a light microscope. Mean ± SE.

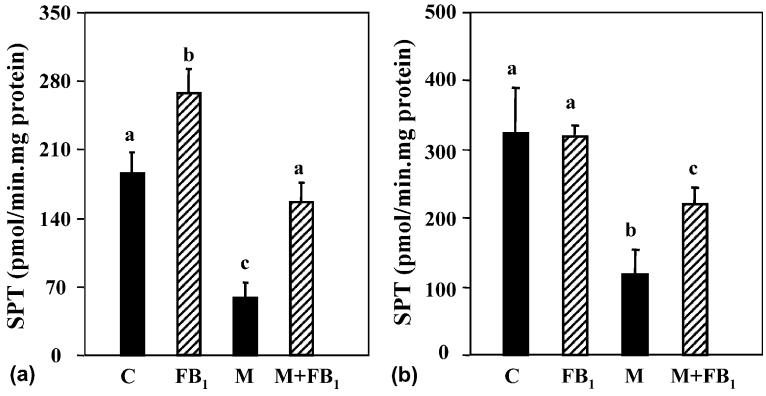

Fig. 4. Myriocin inhibition of serine palmitoyltransferase (SPT) activity in the liver (a) and kidneys (b). Mean ± SE ($n = 5$). Different letters indicate statistical difference at $p < 0.05$.

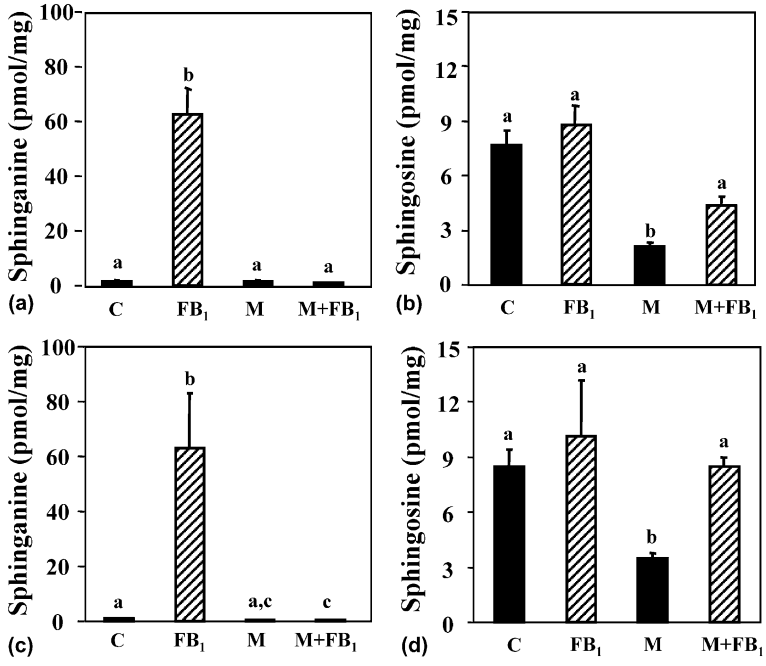

Fig. 5. Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced accumulation of free sphingoid bases, sphinganine and sphingosine, in the liver (a,b) and kidney (c,d). Mean ± SE ($n = 5$). Different letters indicate statistical difference at $p < 0.05$.

Table 2
Concentrations of complex sphingolipids and sphingomyelin in the mouse liver after myriocin and FB_1 treatment[a]

Treatment	Complex sphingolipid containing sphingosine[b]	Complex sphingolipid containing sphinganine[b]	Complex sphingolipid containing sphingosine and sphinganine[b]	Sphingomyelin[c]
Control	297.8 ± 12.3[a]	18.4 ± 3.1[a]	312.6 ± 12.2[a]	252.8 ± 14.0[a]
FB_1	67.9 ± 10.5[b,c]	20.2 ± 2.2[a]	90.1 ± 9.0[b]	47.9 ± 12.8[b]
Myriocin	70.0 ± 2.3[b]	5.2 ± 3.4[b]	74.2 ± 3.4[b]	128.8 ± 9.5[c]
Myriocin + FB_1	56.1 ± 2.0[c]	6.1 ± 1.4[b]	60.9 ± 2.9[c]	30.1 ± 9.6[b]

[a] The values of sphingolipids and sphingomyelin are all expressed as mean ± SE (pmol/mg wet tissue, $n = 5$). Different letters besides the values indicate significant difference at $p < 0.05$.
[b] Sphingolipids were extracted by acid hydrolysis and determined by HPLC. Complex sphingolipids estimated by the differences between total complex sphingolipids (acid hydrolyzed lipid extracts) and free sphingoid (base hydrolyzed lipid extracts) bases.
[c] Determined by enzyme-coupled Amplex® Red assay.

decreased by FB_1, myriocin, or myriocin + FB_1, with the greatest reduction by myriocin + FB_1.

3.4. Myriocin reversed FB_1-induced expression of selected cell signaling factors

FB_1 significantly increased expression of TNFα, TNFR1, TRAIL, LTβ, IFNγ and TGFβ1 (Fig. 6). Myriocin alone did not alter the constitutive levels of expression for these genes in the liver; however, it effectively blocked the FB_1-induced overexpression of all the above genes.

4. Discussion

The well studied biochemical effect of FB_1 is inhibition of ceramide biosynthesis with resultant accumulation of free sphinganine (Wang et al., 1991). Many downstream and concurrent effects resulting from exposure to FB_1 have been demonstrated including increases in production of various cell signal factors (Bhandari et al., 2002; Bhandari and Sharma, 2002). In the present study, we found that myriocin significantly prevented the accumulation of free sphinganine and reversed the induction of selected cell signal factors in the TNFα signal pathway and other cytokines in response to FB_1, but had minimal or no protective effect on FB_1-induced increase in activities of plasma ALT and AST and the number of apoptotic hepatocytes. In fact, a five-day combination of FB_1 and myriocin had an additive, or even likely synergistic effect leading to lethality of these animals.

Myriocin prevented FB_1-induced cell death in vitro in LLC-PK_1 cells, a porcine kidney epithelial cell line (He et al., 2002a; Riley et al., 1999a,b), and HT29 cells, a human colonic cell line (Schmelz et al., 1998). However, our findings in the current in vivo study showed that myriocin did not significantly prevent FB_1-induced liver damage even though it has been shown that there is a

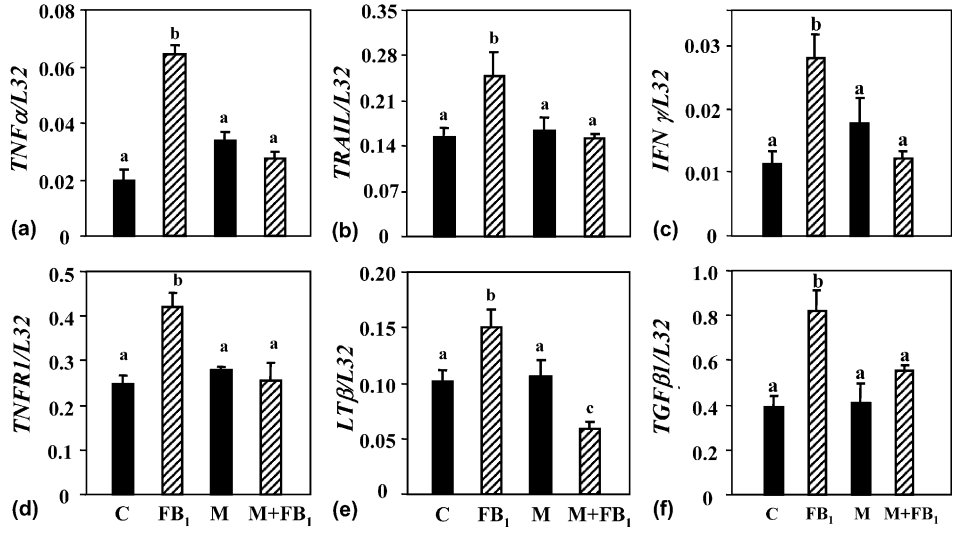

Fig. 6. Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced expression of (a) *tumor necrosis factor* (TNF)α, (b) TNF-related apoptosis-inducing ligand (TRAIL), (c) interferon (IFN)γ, (d) TNF receptor (TNFR)1, (e) Lymphotoxin β (LTβ), and (f) transforming growth factor (TGF)β1, analyzed by quantitative ribonuclease protection assay, in mouse liver. Mean ± SE ($n = 5$). Different letters indicate statistical difference at $p < 0.05$.

close correlation between the extent and severity of fumonisin-induced apoptosis/hepatotoxicity and the degree of elevation in free sphinganine, a marker for FB_1 inhibition of ceramide synthase in BALB/c mice (Tsunoda et al., 1998; Riley et al., 2001). While there is a clear protective effect of myriocin on FB_1-induced cell death in short term in vitro studies, demonstration of a protective effect in vivo is not as easily accomplished for several reasons. For example, there is no information on the specifics of the toxicity of myriocin, the kinetics of its distribution, or its peripheral effects. While it is known to be a potent inhibitor of SPT, the first and rate limiting step in de novo sphingolipid biosynthesis, it is also known to be a potent immunosuppressant (Miyake et al., 1995) and its immunosuppressive effects are independent of its ability to inhibit SPT (Fujita et al., 1996).

The use of myriocin to prevent the sphinganine accumulation and subsequent FB_1 toxicity, as done in this study, was not successful. In fact, the combination of myriocin and FB_1 was highly toxic after five daily treatments for the survival in female BALB/c mice. There was 100% mortality after five daily treatments of myriocin plus FB_1 and yet after three days of exposure to the combination there was no evidence of increased hepatotoxicity. Present data show that levels of complex sphingolipids and sphingomyelin were reduced by FB_1 and myriocin, and the reduction was greater by myriocin plus FB_1 than by either agent alone. It appears that the combined effects of myriocin on the biosynthesis of more complex sphingolipids and sphingomyelin was the cause of the increased toxicity at five days or the lack of a protective effect at three days. Inhibition of ceramide synthase in response to FB_1 results not only in the accumulation of free sphingoid bases but also in the reduction of ceramide and complex sphingolipids (Tolleson et al., 1999; Wang et al., 1992; Yoo et al., 1996). Myriocin potently inhibits the activity of SPT, and thereby blocks biosynthesis of free sphinganine, the precursor of de novo synthesized ceramide (Hannun et al., 2001). The reduction of complex sphingolipids by the combination of FB_1 and β-chloroalanine, a nonspecific inhibitor of SPT, was greater than that mediated by either compound alone in LLC-PK_1 cells (Yoo et al., 1996). Thus, under our present in vivo study conditions, myriocin probably had an additive effect on decreased complex sphingolipid and sphingomyelin biosynthesis, and thereby depletion of complex sphingolipids and sphingomyelin occurred more rapidly in mice treated with combination of myriocin and FB_1 than that in either FB_1 or myriocin-treated mice. Sphingolipids and their metabolites mediate a variety of cell signaling processes involved in cell growth, apoptosis, and proliferation (Ohanian and Ohanian, 2001). Depletion of sphingolipids disrupts cell functions (Hidari et al., 1996). Previous studies have shown that elevated free sphingoid bases as well as depletion of ceramide and complex sphingolipid contributed to FB_1 cytotoxicity in cell cultures (Tolleson et al., 1999; Yoo et al., 1996). It is possible that myriocin facilitated the decrease in biosynthesis of ceramide and complex sphingolipid as well as sphingomyelin, and thus potentiated disruption of downstream effects that are dependent on more complex sphingolipids and sphingomyelin. However, myriocin may have some biological effects independent of inhibiting SPT activity, such as potent immunosuppressive action (Fujita et al., 1996)

Free sphinganine and sphingosine can be metabolized to sphinganine/sphingosine-1-phosphate by sphingosine kinase (Maceyka et al., 2002; Merrill et al., 2001). Accumulation of free sphingoid bases following FB_1 exposure results in accumulation of intracellular sphingoid base-1-phosphates (Merrill et al., 2001). It is possible that sphinganine-1-phosphate accumulation in tissues of animals exposed to FB_1 could promote cell survival. While sphingosine-1-phosphate is clearly an important cell survival and anti-apoptotic signaling factor (Maceyka et al., 2002), the possible role of sphinganine-1-phosphate in cell survival in vivo after FB_1 exposure is unknown. It is likely that myriocin reduced the formation of sphingoid base-1-phosphates as a consequence of blocking sphinganine synthesis. As a result, any possible protective effects of sphinganine-1-phosphate in FB_1-treated liver would be prevented.

It has been demonstrated that FB_1 increases expression of many types of genes in different cell signal transduction pathways. For example, treatment of mice with FB_1 increased expression of TNFα, IL12, IFNγ, *c-myc* and many other genes in the liver and kidney (Bhandari and Sharma, 2002; Bhandari et al., 2002). It is becoming apparent that expression of cell signaling genes is related to the disruption of sphingolipid metabolism following FB_1 exposure. In LLC-PK_1 cells, FB_1 induced a transient increase in TNFα expression, while the induction of TNFα was not reduced by myriocin, suggesting accumulation of free sphingoid bases may not be the cause of TNFα induction (He et al., 2001). However, it is possible that sphinganine/sphingosine-1-phosphates resulting from accumulated free sphinganine and sphingosine induce expression of these genes in other cell types following FB_1 exposure. It has been shown that sphingosine-1-phosphate as well as sphinganine-1-phosphate, but not sphingosine or sphinganine, enhanced the secretion of IL-2 and IFNγ via *Pertussis* toxin-sensitive sphingosine-1-phosphate receptors in peripheral T cells stimulated with anti-CD3 plus anti-CD28 (Jin et al., 2003). In the current study, myriocin treatment did not alter constitutive expression of TNFα, TNFR1, TRAIL, LTβ, IFNγ and TGFβ1; FB_1-increased expression of all these genes in liver, while myriocin prevented FB_1-induced increases in expression of these genes. The reversal of FB_1-induced modulation of these genes was concomitant with the reduced accumulation of free

sphingoid bases by myriocin. These results suggest that accumulation of free sphinganine or its metabolites as a result of FB_1 exposure could mediate overexpression of the above hepatic genes.

In conclusion, the current study demonstrated that five day exposure of mice to myriocin plus FB_1 resulted in overall death, indicating that myriocin plus FB_1 is highly toxic and therefore myriocin used under the conditions described in this study would have no therapeutic potential for preventing FB_1 intoxication. However, myriocin efficiently prevented FB_1-induced accumulation of free sphinganine as a consequence of SPT inhibition and it reversed FB_1-induced expression of TNFα superfamily signal molecules and other cytokines to the constitutive level. Results suggest that accumulation of free sphinganine contributed to FB_1-modulated expression of these cell signal factors in liver and depletion of complex sphingolipids as well as sphingomyelin produced liver damage after FB_1 exposure. Dosing regimes of myriocin that minimize its potential toxic effects may be possible. For example, since FB_1 is quickly eliminated, exposure to myriocin for a brief period after cessation of FB_1 exposure could possibly prevent the harmful effects of free sphinganine and signaling factors induced by free sphinganine while avoiding the additive effects of co-exposure to two potent inhibitors of sphingolipid biosynthesis.

After three days of exposure, the levels of complex sphingolipids containing both sphingosine and sphinganine were lower in the FB_1 + myriocin-treated group compared to FB_1 or myriocin alone treated animals. The FB_1-induced increase in sphingoid bases has been shown to be a dose- and time-dependent process and it is apparent that the kinetics of depletion of newly synthesized complex sphingolipids was different in the FB_1 + myriocin group compared to the FB_1 or myriocin only treatment groups. Thus any process that was sensitive to or required the biosynthesis of specific complex sphingolipids would have been affected in a longer period of time in the FB_1 + myriocin group compared to the FB_1 or myriocin only groups. Future studies are required to determine the time-course for depletion of complex sphingolipids and the effects on biosynthesis of specific glycosphingolipid species such as GM1 and glucosylceramide. It is also likely that myriocin has yet undefined effects unrelated to SPT inhibition that may be responsible for the observed synergistic response of myriocin and FB_1 and need to be investigated.

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